Kit-of-parts for use in a prime-boost vaccination strategy to protect cloven-footed animals against foot-and-mouth disease virus infection

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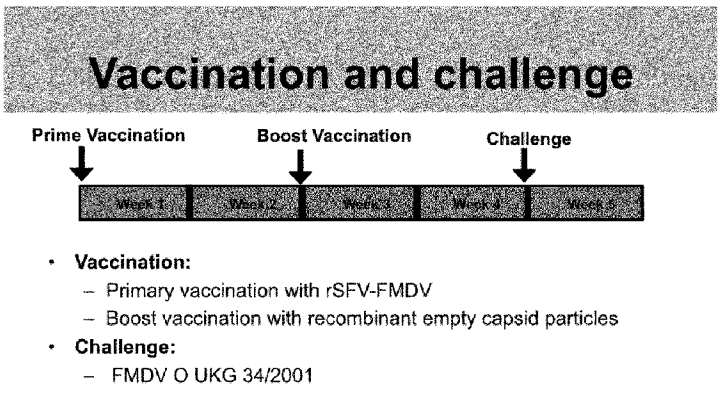
(54) Title: KIT-OF-PARTS FOR USE IN A PRIME-BOOST VACCINATION STRATEGY TO PROTECT CLOVEN-FOOTED ANIMALS AGAINST FOOT-AND-MOUTH DISEASE VIRUS INFECTION

(57) Abstract: The present invention relates to a kit-of-parts for use in immunizing an animal against foot-and-mouth disease virus (FMDV) infection. In particular, the present invention relates to a kit-of-parts containing a priming composition and a boosting composition for use in a prime-boost FMDV-vaccination strategy.

Figure 2



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Kit-of-parts for use in a prime-boost vaccination strategy to protect cloven-footed animals against foot-and-mouth disease virus infection

Technical field of the invention

The present invention relates to a kit-of-parts for use in immunizing an animal against infection by foot-and-mouth disease virus (FMDV). In particular, the present invention relates to a kit-of-parts containing a priming composition and a boosting composition for use in a prime-boost FMDV-vaccination strategy.

Background of the invention

FMDV is a virus that infects animals important for human food production such as cattle, pigs and sheep. It is currently circulating in Africa, Asia and likely South America, but sporadic outbreaks in FMDV-free countries makes it a global problem. The virus is highly contagious and can be transmitted readily via direct contact, contaminated materials and also through the air. This makes current vaccine production a challenge since it requires large scale growth of infectious virus, necessitating vaccine production in special, high containment facilities.

Foot-and-mouth disease (FMD) is generally considered to be the most economically important viral disease of farm animals. It is estimated that FMD costs about US$ 10,000,000,000 annually around the globe (Knight-Jones & Rushton, 2013) and indeed incursions of the disease into single countries which are normally FMD-free but have a highly developed agricultural industry (as in Europe and USA) can result in costs of this magnitude as well. Notably, in the U.K. in 2001, the epizootic affected over 2000 farms and resulted in the slaughter of several million animals; in addition there was prolonged loss of trade in animals and their products plus loss of tourism etc.

Elimination of FMD from Europe was achieved during the 1970/1980's by the effective use of vaccines based on chemically inactivated FMD virus (FMDV) in combination with well-developed veterinary services. Europe no longer allows vaccination against FMDV except when outbreaks occur. It is easier to ensure that animals are free of the virus if they have no anti-FMDV antibodies. However, Europe does maintain a vaccine bank that can supply countries with (inactivated) vaccine when required.

Vaccination against FMDV is used in many countries around the world and billions of doses are produced annually. These are generally based on FMDV that is grown in cell culture, within expensive high containment facilities, and then chemically inactivated prior to use in conjunction with an adjuvant. These vaccines can be useful for the control of the disease but there are various shortcomings including a short duration of immunity, typically about 6 months, a requirement for a cold chain, limited antigenic cross-reactivity etc. (see Belsham & Bøtner, 2015).

Thus, it is considered necessary to revaccinate animals with the inactivated FMDV vaccine at least 2 times per year to ensure protection against disease. Furthermore, it can be difficult to differentiate between vaccinated and infected animals, especially if animals have received multiple doses of vaccine.

FMDV is an icosahedral virus of about 25 nm in diameter, containing a single-stranded RNA molecule consisting of about 8500 nucleotides, with a positive polarity. This RNA molecule comprises a single large open reading frame (ORF), encoding a single polyprotein containing, inter alia, the capsid precursor also known as protein P1-2A. The protein P1-2A is myristylated at its amino-terminal end. During the maturation process, the protein P1 is cleaved by the protease 3C into three capsid proteins known as VP0, VP1 and VP3. The viral capsids may be assembled without the presence of an RNA molecule inside it (so called "empty capsids"). In these empty capsids, cleavage of the VP0 to VP4 and VP2 can occur (as found in the mature virus).

Seven distinct serotypes of FMDV are known (O, A, C, SAT1, SAT2, SAT3 and Asia-1), there is no cross protection between them and thus it is essential that a vaccine of the same virus serotype is used in the face of an outbreak of disease. In general, there is a considerable advantage in having a vaccine which produces the viral antigen within cells (e.g. as with a "live" vaccine) rather than simply administering non-infectious antigen. The most successful vaccines used globally have each been live attenuated viruses (e.g. against smallpox, poliovirus, rinderpest) and these all induce long-term immunity. However it has not been possible to generate safe, effective, live attenuated viruses based on FMDV, in part because of the wide host range of the virus (cloven-footed animals including cattle, pigs, sheep, and about 70 wildlife species).

In Porta et al. (2013b), FMDV-challenge occurred after administration of two doses of recombinant empty FMDV-capsid proteins of serotype A, however all animals that were given unmodified empty capsid particles showed viremia post-challenge.

WO06073431A2 discloses *inter alia* recombinant avipox vectors and viruses that express antigens of FMDV. Also disclosed is a prime-boost immunization or vaccination method of an animal against at least one FMDV antigen comprising administering to the animal e.g. a priming DNA vaccine followed by a boosting composition that comprises the FMDV antigen expressed by e.g. the DNA vaccine.

WO2011112945A2 discloses *inter alia* kits for use in vaccination against FMDV as well as FMDV polypeptides, antigens, epitopes or immunogens that elicit, induce or stimulate a response in e.g. bovines. WO2011112945A2 also discloses a prime-boost method and a kit for performing said method, which can include a recombinant viral vector used to express an FMDV coding sequence followed by the administration of vaccine or composition comprising FMDV antigen.

WO11007339A2 discloses a chimeric FMDV nucleic acid molecule encoding a first FMDV strain, virus or isolate, wherein nucleotides encoding an outer capsid region have been replaced with nucleotides encoding an outer capsid region of a second FMDV strain, virus or isolate which includes or has been modified so as to introduce a heparan sulfate proteoglycan binding site.

US20040001664 discloses FMD vaccine, using as antigen an efficient amount of empty capsids of FMDV. The empty capsids are obtained by expressing, in eukaryotic cells, cDNA of the P1 region of the FMDV genome coding for the capsid and cDNA of the region of the FMDV genome coding for protease 3C.

WO13001285 discloses modified VP1 capsid protein from FMDV. The modified VP1 capsid protein comprises for example an epitope tag, an immunomodulatory polypeptide or a target molecule. WO13001285 also relates to FMDV particles and vaccines, which comprise such a VP1 capsid protein and uses thereof. WO13001285 also discloses a FMDV particle, which is an empty capsid FMDV-like particle.

Thus, the kit-of-parts and the prime-boost strategy of the present invention differs from many of the existing vaccine technology in *inter alia* the following respects:

- no requirement for high containment production facilities
- induces complete block of virus dissemination within challenged animals
- has potential for longer duration of immune response

- can include vaccine marker
- lacks FMDV non-structural proteins (other than $3C^{pro}$) which could simplify differentiation of FMDV vaccinated from infected animals

In light of the above-outlined drawbacks of current FMD-vaccines, there is a need in the art for a FMD-vaccine that:
- does not cause FMDV
- can be produced in standard laboratories without high-containment facilities
- provides longer lasting protection
- can more easily be genetically manipulated to quickly make new vaccines reflecting the current circulating FMDV-strains
- could potentially protect against multiple or even all seven serotypes of FMDV-
- is cheaper to produce
- is stable and can be distributed/produced in countries with warm climate and need for long transportation
- can be distinguished from FMD virus infection in an animal

Summary of the invention

Thus, an object of the present invention relates to prime-boost FMDV-vaccination strategy by using a kit-of-parts comprising a priming composition and a boosting composition.

In particular, it is an object of the present invention to provide a kit-of-parts for use in immunizing an animal against FMDV-infections that solves the above mentioned problems of the prior art.

The inventors have established that a prime-boost vaccination strategy can achieve protection of cattle against FMDV challenge. There are two components used in this kit-of-parts and underlying prime-boost strategy; these are: (i) a single cycle alphavirus vector system expressing FMDV-antigens *in vivo* within cells of the animal plus (ii) a non-infectious preparation of FMDV empty capsids (capsid particles).

Thus, one aspect of the invention relates to a kit-of-parts for use in immunizing an animal against FMDV-infections, wherein the kit-of-parts comprises:
(i) a vessel containing a priming composition which comprises a single-cycle alphavirus vector system expressing FMDV-antigens *in vivo* within cells of the animal;

(ii) a vessel containing a boosting composition which comprises non-infectious FMDV-capsid particles;
for successive administration to the animal wherein the priming composition is administered into the animal prior to the administration into said animal of the boosting composition.

An even further aspect of the invention relates to a prime-boost vaccination against FMDV in an animal, wherein a priming composition, which comprises a single-cycle alphavirus vector system expressing FMDV-antigens *in vivo* within cells of the animal, is administered to the animal prior to a subsequent administration to said animal of a boosting composition, which comprises non-infectious FMDV-capsid particles.

Advantages of the present invention

The prime-boost strategy on which kit-of-parts according to the present invention is based, makes use of a "single-cycle" RNA virus vector which is capable of expressing the FMDV antigens *in vivo* within cells of the animal (priming composition) and primes a surprisingly strong immune response upon subsequent administration of the boosting composition with non-infectious antigen.

High potency vaccines, generated using current technology, can induce sterile immunity (i.e. no virus replication following virus challenge) whereas standard potency vaccines do not and limited virus replication can be detected post-challenge in animals given the lower dose of vaccine.

The results obtained with the prime-boost strategy on which the kit-of-parts of the invention is based, surprisingly indicated that no viremia occurs after FMDV-challenge, which is equivalent to that achieved with the use of the high potency vaccine.

In fact, the prime-boost strategy, on which the kit-of-parts of the invention is based, surprisingly showed a complete block on FMDV-dissemination after administration of the prime and boost compositions of the prime-boost vaccinations as well as a complete absence of FMDV in serum post FMDV-challenge, i.e. within challenged animals.

Hitherto, no "live" vaccine against FMDV has proved suitable for use. However, the single-cycle alphavirus of the priming composition of the invention, such as Semliki Forest Virus (SFV)-FMDV, does result in the production of the FMDV-capsid proteins within cells (as would occur with a "live" virus rather than the

antigen only being presented as an inactivated product) and this may elicit an immune response of a different nature than the current, inactivated vaccine.

The level of anti-FMDV antibodies elicited by the prime-boost strategy was significantly higher than that generated by a single FMDV-challenge of naïve animals with infectious virus.

A further advantage of the kit-of-parts of the present invention compared to the current FMDV-vaccines, is the ease of modifying the sequences of the priming- and/or boosting compositions in a wide range of different ways, e.g. to improve capsid stability, diversify antigenicity (to give protection against different strains) and to add specific markers which can be used to differentiate infected from vaccinated animals (DIVA).

Other advantages of the prime-boost vaccination kit of the present invention are the potential for longer duration of immune response, inclusion of vaccine marker and lack of FMDV non-structural proteins, which in turn simplifies the differentiation between FMDV-vaccinated and infected animals.

With the currently used conventional FMDV vaccines, it is considered necessary to revaccinate animals with the inactivated FMDV vaccine at least 2 times per year to ensure protection against disease since the duration of immunity generated by these inactivated virus antigens is short lived.

The prime-boost immunization protocol of the invention uses an infectious virus (albeit single cycle infection) in the priming step, which results in the production of the FMDV capsid proteins within the animal cells. This will generate a distinct immune response from that achieved using inactivated antigens. It is expected that this will achieve long-term immunity (c.f. "live" vaccines used to protect against poliovirus, measles virus, mumps virus and rubella virus).

In addition, since the recombinant SFV vectors containing FMDV inserts (termed rSFV-FMDVs) vectors produce FMDV antigens within cells then the nature of the immune response generated by this two-step strategy may be expected to have improved characteristics compared to that elicited by conventional, inactivated, FMDV vaccines. In particular, the cell-mediated arm of the immune system may be stimulated more efficiently by the intracellular expression, processing and presentation on the cell surface of viral antigens. This may be particularly relevant with respect to the duration of immunity.

The priming composition

The kit-of-parts of the invention relies on the use of a well-established "split helper" single-cycle viral vector system which preferably is based on SFV (Figure 1) to express the correctly processed FMDV-capsid proteins (antigen) within cells of the animal as a priming step within a two stage prime-boost vaccination strategy (see Figure 2).

As illustrated in Figure 1, the "split helper" single-cycle viral vector system, provides the production of the SFV-capsid proteins from two separate RNA-transcripts whereas within the SFV they are produced from a single RNA-transcript. These transcripts are not packaged into the recombinant SFV (rSFV)-viruses and the potential for recombination between the SFV-capsid coding sequences and the modified rSFV is greatly diminished by "splitting" the capsid coding sequences into two transcripts.

rSFV vectors containing three different types of FMDV-cassette (P1-2A, P1-2A-3CC142S and P1-2A-mIRES-3C) have been made (Figure 3). They have all been tested in cells, in culture, and shown to express the anticipated FMDV proteins that are required to induce an anti-FMDV immune response.

The P1-2A-mIRES-3C construct produces two proteins independently, i.e. P1-2A and 3Cwt. The mIRES region does not encode any protein itself but allows two separate proteins to be made from one mRNA transcript.

The FMDV-particle consists essentially of a protein shell (capsid) around a single copy of the RNA genome. The capsid consists of 60 copies of 4 different proteins, termed VP1, VP2, VP3 and VP4.

The VP1, VP2 and VP3 make up the outer surface of the capsid while VP4 is located internally within the FMDV-particle. Each of the viral proteins is produced from a single polyprotein. The capsid protein precursor is termed P1-2A, as described and shown in Figure 3, which is processed by the virus encoded 3C protease ($3C^{pro}$) to make VP1, VP3 and VP0 (the precursor of VP2 and VP4). Processing of the VP0 to VP2 and VP4 occurs either on encapsidation of the viral RNA or following empty capsid assembly.

The inventors have used a viral vector system to express the optimized P1-2A+$3C^{pro}$ cassettes and shown that the expected protein expression occurs within infected cells.

rSFVs containing three different FMDV cassettes have been made (see Figure 3) and are here termed rSFV-FMDVs. These cassettes either encode the capsid precursor (P1-2A) alone or with the $3C^{pro}$ (the level of $3C^{pro}$ activity is reduced by two separate means in the two cassettes rSFV-FMDV-P1-2A-3CC142S and rSFV-FMDV-P1-2A-mIRES-3C).

RNA transcripts from these vectors can be packaged by SFV capsid proteins, if these are co-expressed by helper RNAs, and this generates infectious virus particles that can only initiate a single round of infection.These packaged rSFV-FMDVs have each been tested in cells and shown to express the anticipated FMDV proteins (Figure 4 (top)) and the fully processed capsid proteins (but not the P1-2A precursor alone) have been found to self-assemble into empty capsid particles, as expected (see Figure 4 (middle)).

The packaged rSFVs are also infectious for animals but following inoculation into animals, no transmission within the animal or to other animals occurs and they do not cause any disease.

More specifically, the FMDV cDNA cassettes used, that encode the capsid precursor (P1-2A) alone or with $3C^{pro}$ are shown in Figure 3 and were prepared by standard methods (Sambrook et al., 1989). The split-helper RNA system for production of rSFV particles as shown in Figure 1 was based on pSFV3, pSFV-helper-C-S219A and pSFV-helper-S2 vectors (Smerdou and Liljeström, 1999). The three plasmids containing the different FMDV cDNA inserts were digested with EcoRI and XmaI to release the FMDV cDNA cassettes, and the pSFV3 vector was digested with BglII and XmaI or with BglII and EcoRI (the pSFV3 vector contains three EcoRI sites). The pSFV3-O-P1-2A, pSFV3-O-P1-2A-3CC142S and pSFV3-O-P1-2A-IRESgtta3Cwt (here referred to as pSFV3-O-P1-2A-mIRES-3C) were made through a single-step three-part ligation (between the different EcoRI/XmaI fragments and two parts of the backbone vector, i.e. a XmaI/BglII part and a BglII/EcoRI part). Plasmids were amplified in *Escherichia coli* (Top10, Invitrogen), purified (Midiprep kit, Fermentas) and verified by sequencing.

The boosting composition

The second boosting step of the prime-boost FMDV-vaccination strategy on which the kit-of-parts of the invention relies, has been tested using non-infectious FMDV-empty capsid particles produced using e.g. a vaccinia virus based system.

As mentioned above, the FMDV-empty capsid consists of 60 copies of 3 different proteins, termed VP1, VP3 and VP0 proteins. These proteins can "self-assemble" within cells of animals to make empty capsid particles, which appear very similar to intact virus particles, but lack the RNA genome and hence are completely non-infectious. Cleavage of VP0 to VP2 and VP4 can occur and thus the particles may contain both unprocessed VP0, the precursor of VP4 and VP2, as well as the mature products.

It should be noted that conventional FMDV vaccine preparations could potentially be used for the boosting step in the process described here and the combination of inactivated FMDV antigen with the primary vaccination using the rSFV-FMDV vector may result in an enhanced immune response. This approach could be a useful "half-way house" between the use of current technology and full adoption of materials generated outside of high containment. In addition, it may serve to extend the duration of immunity achieved compared to that obtained with the conventional vaccine alone.

Description of the figures

Figure 1 shows a schematic representation of the SFV split helper system (Smerdou and Liljestrom, 1999).

Figure 2 shows the concept of the prime-boost FMDV-vaccination and challenge strategy on which the present invention is based, i.e. primary vaccination with a single-cycle alphavirus vector system expressing FMDV-antigens within cells of the animal (priming composition), e.g. rSFV-FMDV, followed by a boost vaccination with vaccinia virus expressed, non-infectious, recombinant FMDV-capsid particles. Challenge: FMDV O UKG 34/2001.

Figure 3 shows a schematic representation of the FMDV genome and the rSFV plasmids used in this study. The P1-2A, P1-2A-3CC142S and P1-2A-mIRES-3C FMDV cDNA cassettes have been described elsewhere (Polacek et al., 2013; Gullberg et al., 2013a) and the pSFV3 and split helper plasmids have been described by Smerdou & Liljeström (1999). Positions of relevant restriction enzyme sites used are shown. Abbreviations: P1-2A: capsid precursor protein; 3C: $3C^{pro}$ wild-type; mIRES: internal ribosome entry site GTTA mutant; SP6: SP6 promotor; nsP1-P4: non-structural proteins 1-4; PS: packaging signal; 26S: 26S subgenomic promotor; C: capsid; p62, 6K and E1: spike proteins.

Figure 4 (top): It has been demonstrated in cells that the rSFV vector containing the FMDV P1-2A cassette produces the P1-2A capsid precursor as an approx. 90kDa protein. When the FMDV-cassette encodes the FMDV-capsid precursor together with the 3C protease then processing of the P1-2A to VP0, VP3 and VP1 (and 2A) occurs. Figure 4 (middle): In order to assess the assembly of the products expressed from the rSFV-FMDVs, infected cell lysates were analysed on sucrose gradients and the presence of FMDV proteins in each fraction was determined by ELISA. The intact P1-2A remains close to the top of the gradient whereas the $3C^{pro}$-processed products (expressed from the P1-2A3CC142S and P1-2A-mIRES-3C cassettes) migrate much further into the gradient consistent with assembly into empty capsid particles. Figure 4 (bottom) shows the ability of the FMDV capsid proteins to bind specifically to the integrin $\alpha_v\beta_6$ (a cellular receptor for FMDV), assessed using an ELISA. The protomers, pentamers and empty capsids were each able to bind specifically to this integrin in a divalent cation dependent manner (binding was blocked in the presence of EDTA).

Figure 5 (left hand panels) shows induction of anti-FMDV antibodies following vaccination (prime on day 0 and boost on day 14) and challenge (on day 28). Optical density percentage (ODP) values below 50% are considered positive for anti-FMDV antibodies. Anti-FMDV antibody titres, on selected days, are shown on the right hand side. No detectable viral RNA was present within the sera of cattle (group 2) that received this treatment (see Figure 6). In contrast, unvaccinated cattle (group 1) had no anti-FMDV antibodies prior to challenge but did seroconvert after challenge although they generated only a relatively low titre of such antibodies (Figure 5, right hand panels)); these animals displayed a high level of viremia post challenge (see Figure 6). Cattle (in group 3) that were inoculated with recombinant non-infectious FMDV-capsids followed by the SFV-FMDV vector had an intermediate immune response (Figure 5) and one calf (C9) showed significant levels of viremia post-challenge (Figure 6) and all the cattle in this group showed a much elevated level of anti-FMDV antibodies post-challenge (Figure 5) which is indicative of significant virus replication.

Figure 6 shows the assessment of FMDV RNA in serum. FMDV RNA in serum was measured by RT-qPCR. Group 1 were unvaccinated. Group 2 were vaccinated at day 0 with rSFV-FMDV (prime) and then on day 14 with non-infectious FMDV-capsid particles (boost). Group 3 were vaccinated at day 0 with FMDV non-infectious capsid particles and then on day 14 with the rSFV-FMDV. All animals were challenged with FMDV on day 28. Surprisingly, no detectable virus was present within the sera of cattle (group 2) that received this treatment. Unvaccinated cattle (group 1) developed very high levels of viral RNA in serum

and showed clinical disease. Moreover, cattle that were inoculated with recombinant non-infectious capsids followed by the SFV-FMDV vector (group 3) showed intermediate levels of viral RNA in serum and spread of the virus from the site of inoculation occurred.

Figure 7 shows the level of neutralizing anti-FMDV antibodies in serum samples collected on the indicated days from calves 1-9 in experiment 3 (as described for Figure 6). Anti- FMDV antibodies were determined using a virus neutralization assay in porcine IBRS2 cells. Values are given as $\log_2$ of the reciprocal titre values. Values below the horizontal bar are considered negative whereas values above the bar are considered positive. Intermediate values are considered "inconclusive". It is clear that the prime-boost treatment (given to calves 4-6) generated high levels of neutralizing anti-FMDV antibodies prior to challenge (on PVD 28) with FMDV.

The present invention will now be described in more detail in the following.

Detailed description of the invention

Definitions

Prior to discussing the present invention in further details, the following terms and conventions will first be defined:

- "Administration into an animal" refers to use of an acceptable (according to veterinary practice) and effective amount of the priming and boosting compositions according to the invention, wherein the administration may be via various routes including, but not limited to, intramuscular (IM), intradermal (ID) or subcutaneous (SC) injection or via intranasal or oral administration. The therapeutic composition according to the invention can also be administered by a needleless apparatus, electroporation, by gene gun or gold particle bombardment or similar methods well-known in the art.

-"Animal" refers to mammals, birds, and the like. Animal or host includes mammals and human. The animal may be selected from the group consisting of equine (e.g. horse), canine (e.g., dogs, wolves, foxes, coyotes, jackals), feline (e.g., lions, tigers, domestic cats, wild cats, other big cats, and other felines including cheetahs and lynx), ovine (e.g. sheep), bovine (e.g. cattle), porcine (e.g. pig), caprine (e.g. goat), avian (e.g. chicken, duck, goose, turkey, quail, pheasant, parrot, finches, hawk, crow, ostrich, emu and cassowary), primate (e.g. prosimian, tarsier, monkey, gibbon, ape), and fish. The term "animal" also includes an individual animal in all stages of development, including embryonic and fetal stages.

-"Antigen" refers to a substance that induces a specific immune response in a host animal. The antigen may comprise a whole organism, killed, attenuated or live; a subunit or portion of an organism; a recombinant vector containing an insert with immunogenic properties; a piece or fragment of DNA capable of inducing an immune response upon presentation to a host animal; a polypeptide, an epitope, a hapten, or any combination thereof. Thus, the term "antigen" further contemplates deletions, additions and substitutions to the amino acid sequence of the antigen, as long as the polypeptide functions to produce an immunological response as defined herein. As is well-known in the art, the term "conservative variation" denotes the replacement of an amino acid residue by another biologically similar residue, or the replacement of a nucleotide in a nucleic acid sequence such that the encoded amino acid residue does not change or is another biologically similar residue. In this regard, particularly preferred substitutions will generally be conservative in nature, i.e., those substitutions that take place within a family of amino acids.

- C1, C2, C3 etc.: refers to host animal (calf) subject numbers.

- Empty capsids: when used herein, refers to non-infectious and/or recombinant FMDV-capsids.

- "FMDV-antigen variants" of the present invention refers to FMDV polypeptides, particularly ovine, bovine, caprine or porcine polypeptides and variants or fragments thereof, having at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, 96%, 97%, 98% or 99% sequence identity to an antigenic polypeptide of the invention.

- "Heterologous" means derived from a genetically distinct entity from the rest of the entity to which it is being compared. For example, a polynucleotide may be placed by genetic engineering techniques into a plasmid or vector derived from a different source, and is a heterologous polynucleotide. A promoter removed from its native coding sequence and operatively linked to a coding sequence other than the native sequence is a heterologous promoter.

- "Immunological response" to a composition or vaccine refers to the development in the host of a cellular and/or antibody-mediated immune response to a composition or vaccine of interest. Usually, an "immunological response" includes but is not limited to one or more of the following effects: the production of antibodies, B cells, helper T cells, and/or cytotoxic T cells, directed specifically to an antigen or antigens included in the composition or vaccine of interest.

- ODP values: Optical Density Percentage (compared to negative control serum).

-"Plasmid" covers any DNA transcription unit comprising a polynucleotide according to the invention and the elements necessary for its *in vivo* expression in a cell or cells of the desired host or target; and, in this regard, it is noted that a supercoiled or non-supercoiled, circular plasmid, as well as a linear form, are intended to be within the scope of the invention. Each plasmid comprises or contains or consists essentially of, in addition to the polynucleotide encoding an FMDV antigen, epitope or immunogen, optionally fused with a heterologous peptide sequence, variant, analog or fragment, operably linked to a promoter or under the control of a promoter or dependent upon a promoter.

- "Purified" as used herein does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified polypeptide preparation is one in which the polypeptide is more enriched than the polypeptide is in its natural environment. That is the polypeptide is separated from cellular components. By "substantially purified" it is intended that such that the polypeptide represents several embodiments at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 98%, or more of the cellular components or materials have been removed. Likewise, the polypeptide may be partially purified. By "partially purified" is intended that less than 60% of the cellular components or material is removed. The same applies to polynucleotides. The polypeptides disclosed herein can be purified by any of the means known in the art

- PVD: for example "PVD 0" refers to Post Vaccination Day 0 etc.

- "Recombinant" refers to a polynucleotide semisynthetic, or synthetic origin which either does not occur in nature or is linked to another polynucleotide in an arrangement not found in nature.

_ "rSFV" refers to recombinant Semliki Forest Virus (SFV).

- "Vector" refers to a recombinant DNA plasmid or a DNA or RNA virus that comprises a heterologous polynucleotide to be delivered to a target cell, either *in vitro* or *in vivo*. The heterologous polynucleotide may comprise a sequence of interest for purposes of prevention or therapy, and may optionally be in the form of an expression cassette. As used herein, a vector needs not necessarily be capable of replication in the ultimate target cell or subject. The term includes cloning vectors and viral vectors.

- "Vessel": in the context of the present invention, "vessel" refers to any pharmaceutically and/or acceptable (according to veterinary practice) containers suitable for storing and preserving medication as liquids, powders or capsules, such as vials, multi-dose vials, single-dose vials, phials, flacons, small glass or plastic vessel or bottle, vacutainer, ampoule or similar small sealed vials, etc.

Detailed description of the invention – the animal experiments

In animal experiment 1 (see example 3), following a single inoculation of the rSFVs into cattle, the major target species for vaccination against FMD, an anti-FMDV response was induced that could be detected by ELISA but the level of antibodies produced was relatively low and proved insufficient to protect against challenge with a serotype O FMDV (into the tongue). However, it was apparent that the prior vaccination, with the rSFV-P1-2A-mIRES-3C reduced the duration and level of viremia that occurred following challenge. It was also noted that a much higher level of anti-FMDV antibodies was generated, post-challenge, in the vaccinated animals than in the naïve controls. This suggested that the rSFVs primed the host immune response against the FMDV infection. This effect was observed in animals inoculated with the rSFV-P1-2A and also, separately, with the rSFV-P1-2A-mIRES-3C but the reduction in viral RNA in the serum was clearest with the rSFV-P1-2A-mIRES-3C.

In animal experiment 2 (see example 4), which just focused on the use of the rSFV-P1-2A-mIRES-3C, it was found that a second inoculation with this same virus did not significantly improve the host immune response against FMDV. It may be that the host response to the SFV particles induced by the primary vaccination blocked the ability of the rSFV to boost the anti-FMDV response. No circulation of the rSFV was detected (by RT-qPCR) in the serum of any of the animals following the 2nd vaccination, while it was detected in nearly all animals following the primary inoculation. The vaccinated animals were not protected against FMDV infection either from direct inoculation or by a more natural route of infection from another infected animal. However, once again a strong priming response was apparent. This raised the potential utility of performing a two-stage vaccination process using the rSFV-FMDV in conjunction with empty capsid particles produced by another viral vector.

In animal experiment 3 (see example 5), it has now been shown that administration of the empty capsid particles after the initial inoculation with the rSFV-FMDV particles gave rise to a high level of anti-FMDV antibodies, prior to challenge, which completely suppressed dissemination of the virus from the site of inoculation. Moreover, no lesions were observed away from this site. It is

noteworthy that the order of the inoculations is very important; the administration of the FMDV empty capsids followed by the rSFV-FMDV gave a much less effective immune response than the reverse order.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs.

It should be noted that embodiments and features described in the context of one of the aspects of the present invention also apply to the other aspects of the invention.

In an embodiment, the subject matter disclosed herein is directed to a kit-of-parts for use in immunizing an animal against FMDV infections, wherein the kit-of-parts comprises:
(i) a vessel containing a priming composition which comprises a single-cycle alphavirus vector system expressing FMDV-antigens *in vivo* within cells of the animal;
(ii) a vessel containing a boosting composition which comprises non-infectious FMDV-capsid particles;
for successive administration to the animal wherein the priming composition is administered into the animal prior to the administration into said animal of the boosting composition.

In another embodiment the subject matter disclosed herein is directed to a kit-of-parts for use in immunizing an animal against FMDV infections derived from serotype O, wherein the kit-of-parts comprises:
(i) a vessel containing a priming composition which comprises a SFV system expressing a FMDV-antigen encoded by SEQ ID NOs: 8 (P1-2A-mIRES-3C) *in vivo* within cells of the animal;
(ii) a vessel containing a boosting composition which comprises non-infectious FMDV-capsid particles;
for successive administration to the animal wherein the priming composition is administered into the animal prior to the administration into said animal of the boosting composition.

In still another embodiment the subject matter disclosed herein is directed to the use of the kit-of-parts of the invention wherein the dose of the infectious single cycle viral vector of the priming composition per 100 kg animal is between: $1x10^6$ and $1.5x10^9$ infectious units, $5x10^6$ and $1.5x10^9$ infectious units, $1x10^7$ and

$1.5x10^9$ infectious units, $5x10^7$ and $1.5x10^9$ infectious units, $1x10^8$ and $1.5x10^9$ infectious units, preferably $2x10^8$ and $1.3x10^9$, more preferably $3x10^8$ and $1.1x10^9$, more preferably $4x10^8$ and $1x10^9$, more preferably $5x10^8$ and $9x10^8$, more preferably $5x10^8$ and $8x10^8$, more preferably $5x10^8$ and $7.5x10^8$, most preferably $7.5x10^8$;

and/or

the dose of the non-infectious FMDV-capsid particles of the boosting composition is between 1-20 µg/per 100 kg animal, preferably 2-19 µg/per 100 kg animal, more preferably 3-18 µg/per 100 kg animal, more preferably 4-17 µg/per 100 kg animal, more preferably 5-16 µg/per 100 kg animal, more preferably 4-15 µg/per 100 kg animal, more preferably 5-14 µg/per 100 kg animal, more preferably 6-13 µg/per 100 kg animal, more preferably 7-12 µg/per 100 kg animal, more preferably 6-11 µg/per 100 kg animal, most preferably ca. 10 µg/per 100 kg animal

and/or

the duration between the successive administration of priming composition and boosting composition into the animal is between 10-20 days, preferably 11-19 days, 12-18 days, 13-17 days, 14-16, 14-15 days, most preferably 14 days.

and/or

the route of successive administration into the animal of priming and boosting compositions of the kit-in parts is by intramuscular and/or intradermal and/or subcutaneous injection or via intranasal or oral administration, preferably by subcutaneous injection.

According to a yet further embodiment of the invention is directed to a prime-boost vaccination against FMDV in an animal, wherein a priming composition, which comprises a single-cycle alphavirus vector system expressing FMDV-antigens *in vivo* within cells of the animal, is administered to the animal prior to a subsequent administration to said animal of a boosting composition, which comprises non-infectious FMDV-capsid particles.

Items

1. Kit-of-parts for use in immunizing an animal against FMDV infections, wherein the kit-of-parts comprises:

(i) a vessel containing a priming composition which comprises a single-cycle alphavirus vector system expressing FMDV-antigens *in vivo* within cells of the animal;
(ii) a vessel containing a boosting composition which comprises non-infectious FMDV-capsid particles;
for successive administration to the animal wherein the priming composition is administered into the animal prior to the administration into said animal of the boosting composition.

2. Kit-of-parts according to item 1, wherein the single-cycle alphavirus vector system is a SFV system.

3. Kit-of-parts according to items 1 or 2, wherein the FMDV-antigens of the priming composition are encoded by one or more nucleotide sequence/sequences selected the group consisting of SEQ ID NO: 4 (P1-2A) and/or SEQ ID NO: 6 (P1-2A-3CC142S) and/or SEQ ID NO: 8 (P1-2A-mIRES-3C) and/or SEQ ID NO: 9 (P1-2A+$3C^{pro}$) and/or SEQ ID NO: 11 (VP0 (VP4 and VP2)) and/or SEQ ID NO: 13 (VP1) and/or SEQ ID NO: 15 (VP2) and/or SEQ ID NO: 17 (VP3) and/or SEQ ID NO: 19 (VP4) and/or SEQ ID NO: 21 (2A) and/or SEQ ID NO: 24 (3Cwt) and/or SEQ ID NO: 26 (pSFV3-O-P1-2A-IRESgtta-3Cwt).

4. Kit-of-parts according to items 1-2, wherein the FMDV-antigens of the priming composition are encoded by a nucleotide sequence having at least 60% sequence identity to SEQ ID NO: 4.

5. Kit-of-parts according to any of items 1-4, wherein the animal to be immunized is a cloven-footed animal such as bovine, ovine, porcine or caprine.

6. Kit-of-parts according to any of items 1-5, wherein the FMDV is derived from serotypes selected from the group consisting of serotype O, A, C, SAT1, SAT2 , SAT3 and Asia-1, preferably serotype O.

7. Kit-of-parts according to any of items 1-6, wherein the non-infectious FMDV-capsid particles of the boosting composition are vaccinia virus expressed empty capsids or baculovirus expressed empty capsids.

8. Kit-of-parts according to any of items 1-7, wherein an adjuvant is used in the boosting composition.

9. Kit-of-parts according to item 8, wherein the adjuvant is a mineral oil adjuvant such as Montanide ISA 201 VG (Seppic) mineral oil adjuvant.

10. Kit-of-parts according to any of items 1-9, wherein the priming composition and/or the boosting composition comprise one or more acceptable (according to veterinary practice) carrier(s), excipient(s), and/or vehicle(s) such as water-in-oil emulsion or oil-in-water emulsion.

11. Kit-of-parts according to any of items 1-10, wherein said kit-of-parts further comprises instructions for the successive administration to the animal wherein the priming composition is administered into the animal prior to the administration into said animal of the boosting composition.

12. Kit-of-parts according to any of items 1-2 or 5-11, wherein the antigen of the infectious single cycle viral vector of the priming composition is one or more of SEQ ID NO: 5 (P1-2A), SEQ ID NO: 7 (P1-2A-3CC142S), SEQ ID NO: 10 (P1-2A+$3C^{pro}$), SEQ ID NO: 12 (VP0), SEQ ID NO: 14 (VP1), SEQ ID NO: 16 (VP2), SEQ ID NO: 18 (VP3), SEQ ID NO: 20 (VP4), SEQ ID NO: 22 (2A) or SEQ ID NO: 25 (3Cwt) or any fragments and/or combinations thereof.

13. Kit-of-parts according to any of items 1-12, wherein the non-infectious FMDV-capsid particles of the boosting composition are recombinant particles.

14. Kit-of-parts according to any of items 1-13, wherein the boosting composition is a conventional FMDV vaccine.

15. Kit-of-parts according to any of items 1-14, wherein the non-infectious FMDV-capsid particles of the boosting composition are partially or substantially purified.

16. Kit-of-parts according to any of items 1-15, wherein the kit-of-parts further includes a vaccine marker and/or lacks FMDV non-structural proteins.

17. Use of the kit-of-parts according to any of items 1-16 for immunizing animals against FMDV-infections, wherein the dose of the infectious single cycle viral vector of the priming composition to be administered to the animal is between $1x10^6$ and $1.5x10^9$ infectious units/per 100 kg animal.

18. Use of the kit-of-parts according to any of items 1-16, wherein the dose of the infectious single cycle viral vector of the priming composition per 100 kg animal is between $1x10^6$ and $1.5x10^9$ infectious units, $5x10^6$ and $1.5x10^9$ infectious units,

$1x10^7$ and $1.5x10^9$ infectious units, $5x10^7$ and $1.5x10^9$ infectious units, $1x10^8$ and $1.5x10^9$ infectious units, preferably $2x10^8$ and $1.3x10^9$, more preferably $3x10^8$ and $1.1x10^9$, more preferably $4x10^8$ and $1x10^9$, more preferably $5x10^8$ and $9x10^8$, more preferably $5x10^8$ and $8x10^8$, more preferably $5x10^8$ and $7.5x10^8$, most preferably $7.5x10^8$.

19. Use of the kit-of-parts according to any of items 1-16 for immunizing animals against FMDV-infections, wherein the dose of the non-infectious FMDV-capsid particles of the boosting composition to be administered to the animal is between 1-20 µg/per 100 kg animal.

20. Use of the kit-of-parts according to any of items 1-16, wherein the dose of the non-infectious FMDV-capsid particles of the boosting composition per 100 kg animal is between 1-20 µg, preferably 2-19 µg, more preferably 3-18 µg, more preferably 4-17 µg, more preferably 5-16 µg, more preferably 4-15 µg, more preferably 5-14 µg, more preferably 6-13 µg, more preferably 7-12 µg, more preferably 6-11 µg, most preferably approx. 10 µg.

21. Use of the kit-of-parts of any of items 1-16 for immunizing animals against FMDV-infections, wherein the priming composition is administered into the animal prior to the administration into said animal of the boosting composition.

22. Use of the kit-of-parts of any of items 1-16 for immunizing animals against FMDV-infections, wherein the duration between the successive administration of priming composition and boosting composition into the animal is between 10-20 days, preferably 14 days.

23. Use of the kit-of-parts of any of items 1-16 for immunizing animals against FMDV-infections, wherein the duration between the successive administration of priming composition and boosting composition into the animal is between 10-20 days, preferably 11-19 days, 12-18 days, 13-17 days, 14-16, 14-15 days, most preferably 14 days.

24. Use of the kit-of-parts of any of items 1-16 for immunizing animals against FMDV-infections, wherein the route of successive administration into the animal of priming and boosting compositions of the kit-in parts is by intramuscular and/or intradermal and/or subcutaneous injection or via intranasal or oral administration, preferably by subcutaneous injection.

25. Use of the kit-of-parts of any of items 1-16 for immunizing animals against FMDV-infections, wherein the period between the prime-boost vaccination with the kit-of-parts according to items 1-15, and a subsequent prime-boost vaccination is 0.5 – 10 years, preferably 0.6 – 8 years, preferably 0.7 – 7 years, preferably 0.8 – 6 years, preferably 0.9 – 5 years, preferably 1 – 4 years, preferably 1.1 – 3 years, preferably 1.2 – 2 years, most preferably ca. 2 years .

26. Use of the kit-of-parts of any of items 1-16 for immunizing animals against FMDV-infections, and using the periods of item 24, wherein the subsequent prime-boost vaccination is according to any of items 1-15.

27. Method of prime-boost vaccination against FMDV in an animal, wherein a priming composition, which comprises a single-cycle alphavirus vector system expressing FMDV-antigens *in vivo* within cells of the animal, is administered to the animal prior to a subsequent administration to said animal of a boosting composition, which comprises non-infectious FMDV-capsid particles.

28. Method of prime-boost vaccination against FMDV in an animal according to item 27, comprising the kit-of-parts according to items 1-16, which can be used for serological differentiation between animals infected with FMDV and animals vaccinated against FMDV.

29. Method of prime-boost vaccination against FMDV in an animal according to item 27, comprising the kit-of-parts according to items 1-16, which can be used to readily differentiate between FMDV-infected animals by the presence and/or amounts of antibodies to the FMDV non-structural protein (NSP 3B, FMDV 3ABC or 3D-specific ELISA) using a commercial ELISA kit (e.g. Prionics).

All patent and non-patent references cited in the present application, are hereby incorporated by reference in their entirety.

The invention will now be described in further details in the following non-limiting examples.

Examples

Both the rSFV vector and the recombinant non-infectious FMDV-capsid particles can be generated outside of high-containment facilities, in contrast to conventional FMDV-vaccines.

Example 1 (Preparation of the priming composition)

The inventors have focused recent efforts on the processing of the capsid precursor (P1-2A) by the 3C protease ($3C^{pro}$) (Polacek et al., 2013). This processing yields the capsid proteins VP0, VP3 and VP1 plus the 2A peptide. Optimal protein expression is achieved when the level of $3C^{pro}$ is reduced relative to the capsid precursor (Polacek et al., 2013). "Self-assembly" of the processed capsid proteins occurs to generate "empty" capsid particles (60 copies of each protein, Gullberg et al., 2013a)). Moreover, the inventors have demonstrated that the cleavage of the VP1-2A junction is not essential for the formation of empty capsid particles (Gullberg et al., 2013b) and indeed infectious "self-tagged" viruses with uncleaved VP1-2A can be made (see e.g. Gullberg et al., 2013b).

Construction of pSFV-FMDV plasmids

The methods used to transcribe capped RNAs *in vitro*, electroporate the RNA into baby hamster kidney (BHK) cells and to produce single-cycle infectious rSFV particles were performed as previously described (Karlsson and Liljeström; 2003, Bøtner et al., 2011; Nayak et al., 2006).

The three serotype O FMDV cDNA cassettes indicated in Figure 3 have previously been described in Polacek et al., 2013; Gullberg et al., 2013a. One cassette encodes the capsid precursor P1-2A (from O1 Manisa) alone while the P1-2A-3CC142S cassette encodes P1-2A linked directly (within a single open reading (ORF) to a mutant form of $3C^{pro}$ with much reduced catalytic activity. The third FMDV cDNA cassette, P1-2A-mIRES-3C, includes two separate ORFs for P1-2A and the wt $3C^{pro}$; the expression of the $3C^{pro}$ is dependent on a mutant form of the FMDV IRES element (termed GTTA) that is highly defective (ca. 10% of wt activity) and therefore only produces a relatively low level of the protease relative to the capsid precursor. Each of these three cassettes has been introduced into the SFV expression vector called pSFV3 (Smerdou and Liljeström, 1999) so that the production of the RNA transcripts containing the FMDV sequences is dependent on the activity of the 26S sub-genomic promoter (Figure 3).

The in vivo self-assembly of FMDV empty capsids expressed by rSFV-FMDVs

The processed products of P1-2A can assemble into empty capsid particles (which sediment at ca. 80S) and have much higher immunogenicity than the unprocessed P1-2A or unassembled pentamers. Thus, in order to assess the assembly of the products expressed from the rSFV-FMDVs, infected cell lysates were analysed on sucrose gradients and the presence of FMDV proteins in each fraction was determined by ELISA. The ELISA results confirmed that the $3C^{pro}$ processed products (expressed from the P1-2A3CC142S and P1-2A-mIRES-3C cassettes)

assembled into empty capsid particles to a much higher degree than intact P1-2A (see also Figure 4).

Binding abilities

The ability of the FMDV capsid proteins to bind specifically to the integrin $\alpha_v\beta_6$ (a cellular receptor for FMDV) was also assessed using an ELISA. The protomers, pentamers and empty capsids were each able to bind specifically to this integrin in a divalent cation dependent manner (binding was blocked in the presence of EDTA) (see figure 4).

Example 2 (Preparation of the boosting composition)
Preparation of the non-infectious FMDV-capsid particles expressed from recombinant vaccinia viruses.

The recombinant, non-infectious, FMDV empty capsid particles were produced using a vaccinia virus vector system that is only suitable for use in cell culture, see e.g. Abrams et al., 1995; Porta et al., 2013.

The empty capsid particles were produced by dual infection of RK13 cells with vaccinia virus vTF7-3 (Fuerst et al., 1986) and another containing a T7-P1-2A-$3C^{pro}$ O1 Manisa cDNA cassette and then purified by sucrose gradient centrifugation essentially as described by Porta et al., (2013b).

Example 3 (Animal experiment 1 - rSFV-FMDV followed by challenge):
Animal experiment 1 demonstrated that the rSFV-FMDV vectors used were capable of inducing an anti-FMDV antibody response in cattle, however this response was not strong enough to achieve protection against challenge with FMDV. Similarly, simply boosting with a second dose of the rSFV-FMDV (see animal experiment 2, Example 4) also failed to confer protection.

Animal experiment 1 (results): rSFV-FMDV alone followed by challenge gave rise to antibodies against FMDV pre-challenge but did not protect against FMDV.

In animal experiment 1, eight calves of 2-6 months of age (Danish Jersey calves, approx. 100 kg) were divided into three groups, one group with two animals (control group 1, animals C1 and C2) and the other two groups with three animals in each (group 2, animals C3, C4 and C5 and group 3, animals C6, C7 and C8). Group 2 (C3-C5) and 3 (C6-C8) were vaccinated subcutaneously with $5x10^8$ infectious units of each rSFV expressing FMDV P1-2A or FMDV P1-2A-mIRES-3C,

respectively. The control group received injections of PBS. The procedure used to challenge the cattle has previously been described in Stenfeldt et al., 2011. Briefly, in week three (post vaccination day (PVD) 21), the animals were challenged by sub-epithelial injection, in the tongue, with ca. 10^6 $TCID_{50}$ (in total, as determined in pBTY cells; primary bovine thyroid cells) of FMDV O UKG 34/2001. All animals were monitored daily, with measurements of rectal temperature and observation of clinical signs (drooling and appearance of lesions in the mouth and on the feet). Serum samples were collected at predetermined times until PVD 30 when the experiment was terminated.

Example 4 (Animal experiment 2 - rSFV-FMDV followed by rSFV-FMDV followed by challenge).

Animal experiment 2 (results): rSFV-FMDV + rSFV-FMDV also gave rise to antibodies against FMDV but did still not fully protect against FMDV. However, as in the animal experiment 1 (example 3), it was apparent that following challenge a large increase in antibody titres against FMDV occurred. These reached a level much higher than in the unvaccinated calves.

In animal experiment 2, thirteen calves of 2-6 months of age (Danish Jersey calves, approx. 100 kg) were divided into 5 groups. The control groups, 1 (C1-C2) and 2 (C3-C4), each consisted of two cattle while the test groups 3 (C5-C7), 4 (C8-C10) and 5 (C11-C13) each comprised 3 animals. Cattle in groups 3, 4 and 5 each received $7.5x10^8$ infectious units of the rSFV-P1-2A-mIRES-3C on PVD 0 and then again on PVD 14. Animals in groups 1 and 3 were inoculated into the tongue with FMDV O UKG 34/2001 (ca. 10^6 $TCID_{50}$) on PVD 28. Animals in group 2 (C3-C4, unvaccinated) and group 5 (C11-C13, twice vaccinated) were kept in close contact with those from group 1 (C1-C2) from one day after challenge (PVD 29) in one stable while cattle in group 4 (C8-C10) were kept in contact with the vaccinated and inoculated cattle (C5-C7) in group 3 within another stable. All animals were monitored daily and blood samples were collected at pre-determined times until day 42 when the experiment was terminated.

Outline of the experimental setup
Animals: thirteen in total, 2-6 month old Danish Jersey calves, approx. 100 kg
Duration of experiment: 6 weeks

Vaccination
Week 1 (primary vaccination)
Group 1-2: control groups, two animals/group (no vaccination)

Group 3-5: vaccination groups with 3 animals/group: rSFV-P1-2A-mIRES-3C dissolved in PBS (dose: $5x10^8$ particles/ml, in total 1,5ml subcutaneous injection in neck, in total 3 animals per group)

Week 3 (booster vaccination)
Group 1-2: control groups (no vaccination)
Group 3-5: vaccination groups: rSFV-P1-2A-mIRES-3C dissolved in PBS (dose: $5x10^8$ particles/ml, in total 1,5ml subcutaneous injection in neck, in total 3 animals per group)

Week 5 (inoculation/challenge)
Group 3-5: vaccination groups: Inoculation with FMDV O UKG 34/2001. Dose: 10^6 $TCID_{50}$/animal, in total 1 ml for intra-/subdermal injection in the tongue. The animals are anesthetized (with a combination of Zoletil 50 Vet and Rompun Vet) before inoculation. The day following the inoculation, the animals are moved such that inoculated animals and contact-animals are intermingled.

Blood samples are taken on days: 0, 1, 2, 3, 4, 7, 14, 15, 16, 17, 18, 21, 28, 29, 30, 31, 32, 34, 35, 39 and 42. In total: 22 samples per animal.

Swab samples are taken on days: 0, 28, 29, 30, 31, 32, 34, 35, 37, 39 and 42. In total 11 samples per animal.
Daily monitoring of animals (clinical symptoms, temperature etc.)

Post mortem
All animals of the experiment are sacrificed on day 43 (Pentobarbitalnatrium 30%).

Example 5 (Animal experiment 3 - rSFV-FMDV followed by non-infectious, empty FMDV-capsids followed by challenge)

In animal experiment 3, a two-stage vaccination approach in which the rSFV-FMDV is used as a priming step within a two stage "prime-boost" vaccination strategy (see Figure 1). The second boosting step has been tested using recombinant, non-infectious, FMDV empty capsid particles produced using a vaccinia virus vector system that is only suitable for use in cell culture (see e.g. Abrams et al., 1995; Porta et al., 2013). It has now been shown that the initial inoculation with the rSFV-FMDV vector primes the immune system and leads to a surprisingly large stimulation of the immune response that occurs in cattle

following subsequent inoculation with the boosting antigen. This combination of a "live" single- cycle virus vector followed by inactive antigen results in a complete block on FMDV dissemination within the natural host (as judged by level of FMDV RNA in serum) following later challenge with infectious FMDV (see Figure 6). The efficacy of this approach has been demonstrated within cattle, the most important host species for FMDV.

The level of anti-FMDV antibodies elicited by the prime-boost strategy is better than that generated by a single challenge of naïve animals with infectious FMD virus. Furthermore, the complete absence of FMDV in serum, post-challenge, in the vaccinated animals is a marked improvement on the outcome observed in animals given two doses of the recombinant empty capsids (Porta et al., 2013). In that study, all animals given the unmodified empty capsid particles showed viremia post-challenge.

In the animal experiment 3, nine calves of 2-6 months of age (Danish Jersey calves, approx. 100 kg) were divided into 3 groups. The control group 1 (C1-C3) was unvaccinated while the group 2 (C4-C6) was inoculated with rSFV-P1-2A-mIRES-3C on PVD 0 and then boosted on PVD 14 with ca. 10 µg of O1 Manisa empty capsid particles with Montanide ISA 201 VG (Seppic) mineral oil adjuvant. Group 3 received the same inoculations but in the opposite order, thus the animals were inoculated with the O1 Manisa empty capsid particles in adjuvant on PVD 0 and then with the rSFV-P1-2A-mIRES-3C on PVD 14. The empty capsid particles were produced by dual infection of RK13 cells with vaccinia virus vTF7-3 (Fuerst et al., 1986) and another containing a T7-P1-2A-3C^{pro} O1 Manisa cDNA cassette and then purified by sucrose gradient centrifugation essentially as described by Porta et al., (2013b). All animals were challenged on PVD 28 by needle inoculation into the tongue (as in animal experiment 1, see above).

The presence of FMDV RNA and anti-FMDV antibodies in the cattle serum samples were determined by RT-qPCR (targeting the 5'-UTR) and a blocking ELISA, respectively. The level of viral RNA detected in serum samples was converted to the number of genome copies by reference to a standard curve of reference RNA samples, assayed in parallel, as described previously (Bøtner et al., 2011). Selected serum samples were titrated in the blocking ELISA, using 2-fold dilutions from an initial 1:5 dilution. The titre is the reciprocal of the lowest dilution giving a positive signal. The presence of neutralising anti-FMDV antibodies in sera was determined using virus neutralization assays (VNTs) using O1 Manisa FMDV (ca. 100 $TCID_{50}$) in IBRS2 cells. Two-fold dilutions of sera (starting from 1:4 dilution to 1:512) were tested in 4 separate wells for each dilution and cytopathic effect was

read 3 days post –infection. Results are expressed as VNT titres that are the reciprocals of the serum dilution giving 50% neutralization. Known positive and negative control sera were analysed in parallel and gave values within the expected range.

Outline of the experimental setup
Animals: 9 animals in total, 2-6 month old Danish Jersey calves, approx. 100 kg
Duration of experiment: 6 weeks

The animals were treated as follows:
Group 1: 3 animals, C1-C3 (control, no vaccine and challenge)
Group 2: 3 animals, C4-C6 (rSFV-FMDV vaccine followed by vaccinia virus expressed FMDV non-infectious capsids and challenge)
Group 3: 3 animals, C7-C9 (vaccinia virus expressed FMDV non-infectious capsids followed by rSFV-FMDV vaccine and challenge)

Week 1 (primary vaccination)
Group 2 received rSFV-FMDV vaccine (on $T_{PVD} = 0$)
Group 3 received vaccinia virus expressed non-infectious capsid particles (on $T_{PVD} = 0$).

Week 3 (booster vaccination)
Group 2 received vaccinia virus expressed non-infectious capsids (on $T_{PVD} = 14$).
Group 3 received rSFV-FMDV (on $T_{PVD} = 14$).

Week 5 (inoculation/challenge)
Group 1, 2, and 3 received FMDV O UKG 34/2001 (on $T_{PVD} = 28$): Dose: 10^6 $TCID_{50}$/animal, in total 1 ml for intra-/subdermal injection in the tongue. The animals are anesthetized (with a combination of Zoletil 50 Vet and Rompun Vet) before inoculation.

Blood samples are taken on days: 0, 1, 2, 3, 4, 7, 14, 15, 16, 17, 18, 21, 28, 29, 30, 31, 32, 34, 35, 39 and 42. In total: 22 samples per animal.

Swab samples are taken on days: 0, 28, 29, 30, 31, 32, 34, 35, 37, 39 and 42. In total 11 samples per animal.

Clinical and temperature observations daily.

Serum samples (2x5 ml from each animal) on $T_{PVD} = 14$, 15, 16, 17, 18 and 21

Post mortem
All animals of the experiment are sacrificed on day 42 or 43 (T_{PVD} = 42 or 43) (Pentobarbitalnatrium 30%)

The inventors observed that the combined administration in cattle of a "live" single-cycle virus vector (priming composition) followed by recombinant FMDV-capsid particles (boosting composition) in animal experiment 3 results in a complete block on FMDV dissemination within animals following later challenge with infectious FMDV.

Currently only serotype O cassettes have been used in the present invention, but this is the serotype that is responsible for about 75% of the global FMDV-outbreaks. The technology should be applicable to other strains of serotype O and also to other serotypes if the sequence corresponding to the P1-2A capsid precursor is used in the rSFV vector.

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WO11112945A2

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WO11007339A2

US20040001664

WO13001285

Sequence listing

The O1 Manisa sequence (field virus) sequence is available in GenBank with an Accession no. AY593823.1 (Foot-and-mouth disease virus O isolate o1manisa iso87, complete genome) and the sequences SEQ ID NOs: 4-25 listed below are derived from that)

(organism: Semliki Forest Virus)
SEQ ID NO: 1 (pSFV3 (SFV3) (nucleotide))
SEQ ID NO: 2 (pSFV-helper-C-S219A (nucleotide))
SEQ ID NO: 3 (pSFV-helper-S2 (nucleotide))

(organism: O1 Manisa field strain virus of FMDV)
SEQ ID NO: 4 (P1-2A) (O1Manisa, with an ATG codon added at the start) (nucleotide))
SEQ ID NO: 5 (P1-2A (amino acid))
SEQ ID NO: 6 (P1-2A-3CC142S (nucleotide))
SEQ ID NO: 7 (P1-2A-3CC142S (amino acid))
SEQ ID NO: 8 (P1-2A-mIRES-3C (nucleotide))
SEQ ID NO: 9 (P1-2A+$3C^{pro}$ (nucleotide))
SEQ ID NO: 10 (P1-2A+$3C^{pro}$ (amino acid))
SEQ ID NO: 11 (VP0 (VP4 and VP2) (nucleotide))
SEQ ID NO: 12 (VP0 (amino acid))
SEQ ID NO: 13 (VP1 (nucleotide))
SEQ ID NO: 14 (VP1 (amino acid))
SEQ ID NO: 15 (VP2 (nucleotide))
SEQ ID NO: 16 (VP2 (amino acid))
SEQ ID NO: 17 (VP3 (nucleotide))
SEQ ID NO: 18 (VP3 (amino acid))
SEQ ID NO: 19 (VP4 (nucleotide))
SEQ ID NO: 20 (VP4 (amino acid))
SEQ ID NO: 21 (2A (nucleotide))
SEQ ID NO: 22 (2A (amino acid))
SEQ ID NO: 23 (mIRES (nucleotide))
SEQ ID NO: 24 (3Cwt (nucleotide))
SEQ ID NO: 25 (3Cwt (amino acid))
SEQ ID NO: 26 (pSFV3-O-P1-2A-IRESgtta-3Cwt (nucleotide))

Claims

1. Kit-of-parts for use in immunizing an animal against foot-and-mouth disease virus (FMDV) infections, wherein the kit-of-parts comprises:
(i) a vessel containing a priming composition which comprises a single-cycle alphavirus vector system expressing FMDV-antigens *in vivo* within cells of the animal;
(ii) a vessel containing a boosting composition which comprises non-infectious FMDV-capsid particles;
for successive administration to the animal wherein the priming composition is administered into the animal prior to the administration into said animal of the boosting composition.

2. Kit-of-parts according to claim 1, wherein the single-cycle alphavirus vector system is a Semliki Forest Virus (SFV) system.

3. Kit-of-parts according to any of claim 1-2, wherein the FMDV-antigens of the priming composition are encoded by one or more nucleotide sequence/sequences selected the group consisting of SEQ ID NOs: 4 and/or SEQ ID NOs: 6 and/or SEQ ID NOs: 8 and/or SEQ ID NOs: 9 and/or SEQ ID NO: 11.

4. Kit-of-parts according to claim 1-2, wherein the FMDV-antigens of the priming composition are encoded by a nucleotide sequence having at least 60% sequence identity to SEQ ID NO: 4.

5. Kit-of-parts according to any of claims 1-4, wherein the animal to be immunized is a cloven-footed animal such as bovine, ovine, porcine or caprine.

6. Kit-of-parts according to any of claims 1-5, wherein the FMDV infections is derived from serotypes selected from the group consisting of serotype O, A, C, SAT1, SAT2 , SAT3 and Asia-1, preferably serotype O.

7. Kit-of-parts according to any of claims 1-6, wherein the non-infectious FMDV-capsid particles of the boosting composition are vaccinia virus expressed empty capsids or baculovirus expressed empty capsids.

8. Kit-of-parts according to any of claims 1-7, wherein an adjuvant is used in the boosting composition.

9. Kit-of-parts according to claim 8, wherein the adjuvant is a mineral oil adjuvant.

10. The kit-of-parts according to any of claims 1-9, wherein the priming composition and/or the boosting composition comprise one or more acceptable (according to veterinary practice) carrier(s), excipient(s), and/or vehicle(s) such as water-in-oil emulsion or oil-in-water emulsion.

11. Use of the kit-of-parts according to any of claims 1-10 for immunizing animals against FMDV-infections, wherein the dose of the infectious single cycle viral vector of the priming composition to be administered to the animal is between $1x10^6$ and $1.5x10^9$ infectious units/per 100 kg animal.

12. Use of the kit-of-parts according to any of claims 1-11 for immunizing animals against FMDV-infections, wherein the dose of the non-infectious FMDV-capsid particles of the boosting composition to be administered to the animal is between 1-20 µg/per 100 kg animal.

13. Use of the kit-of-parts of any of claims 1-12 for immunizing animals against FMDV-infections, wherein the priming composition is administered into the animal prior to the administration into said animal of the boosting composition.

14. Use of the kit-of-parts according to claim 1-13, wherein the duration between the successive administration of priming composition and boosting composition into the animal is between 10-20 days, preferably 14 days.

15. Method of prime-boost vaccination against foot-and-mouth disease virus (FMDV) in an animal, wherein a priming composition, which comprises a single-cycle alphavirus vector system expressing FMDV-antigens *in vivo* within cells of the animal, is administered to the animal prior to a subsequent administration to said animal of a boosting composition, which comprises non-infectious FMDV-capsid particles.

Figures

5 Figure 1

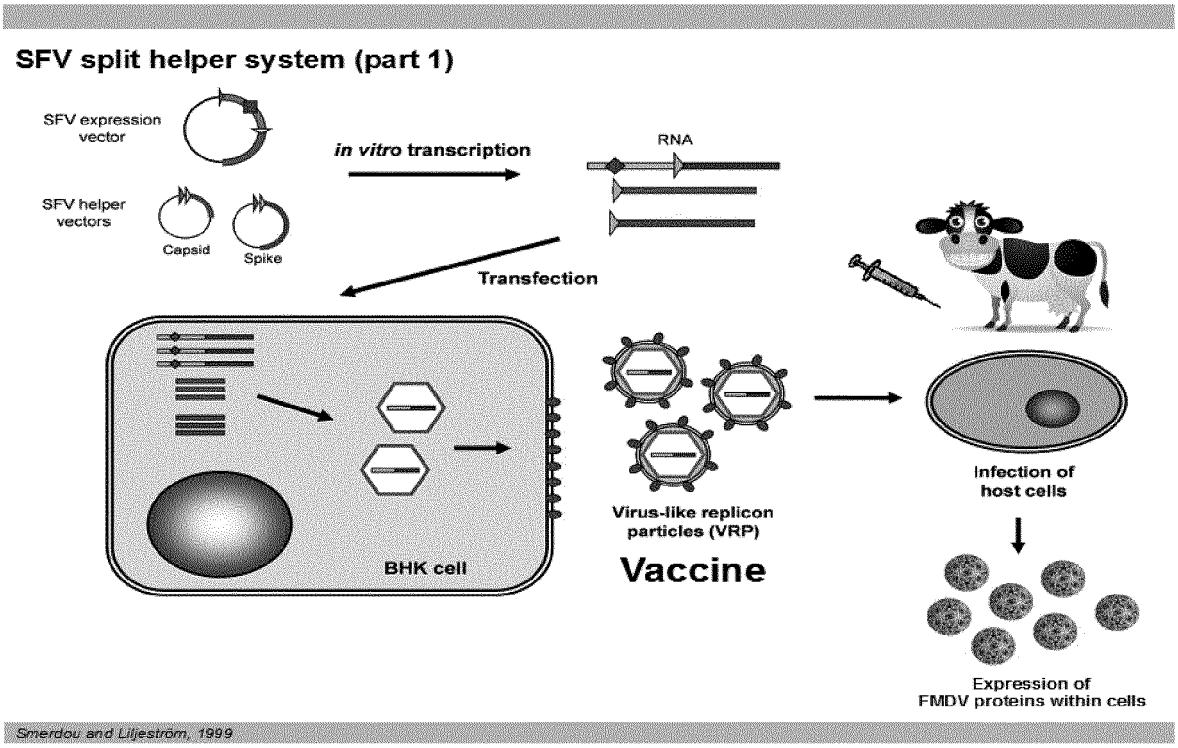


Figure 2

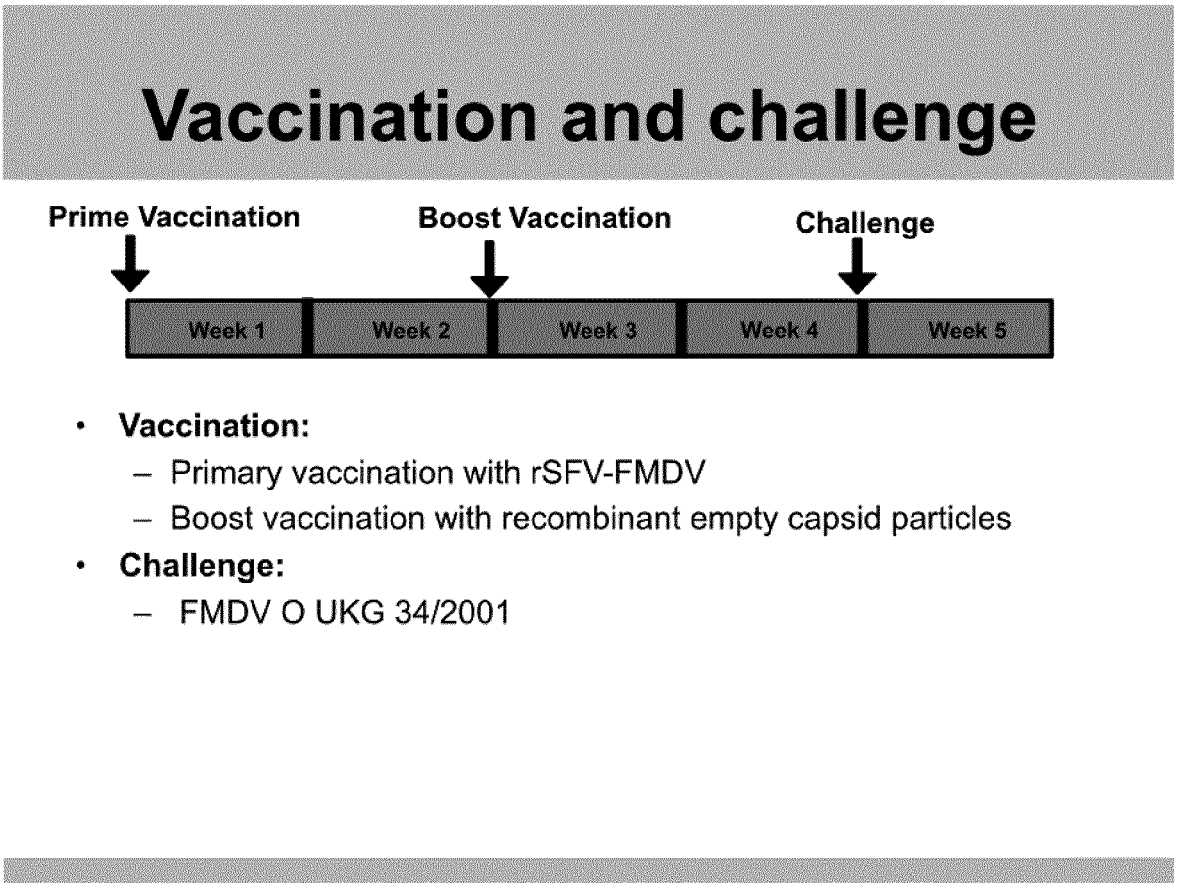

Vaccination and challenge
Prime Vaccination
Boost Vaccination
Challenge
Week 1
Week 2
Week 3
Week 4
Week 5
• Vaccination:
– Primary vaccination with rSFV-FMDV
– Boost vaccination with recombinant empty capsid particles
• Challenge:
– FMDV O UKG 34/2001

Figure 3

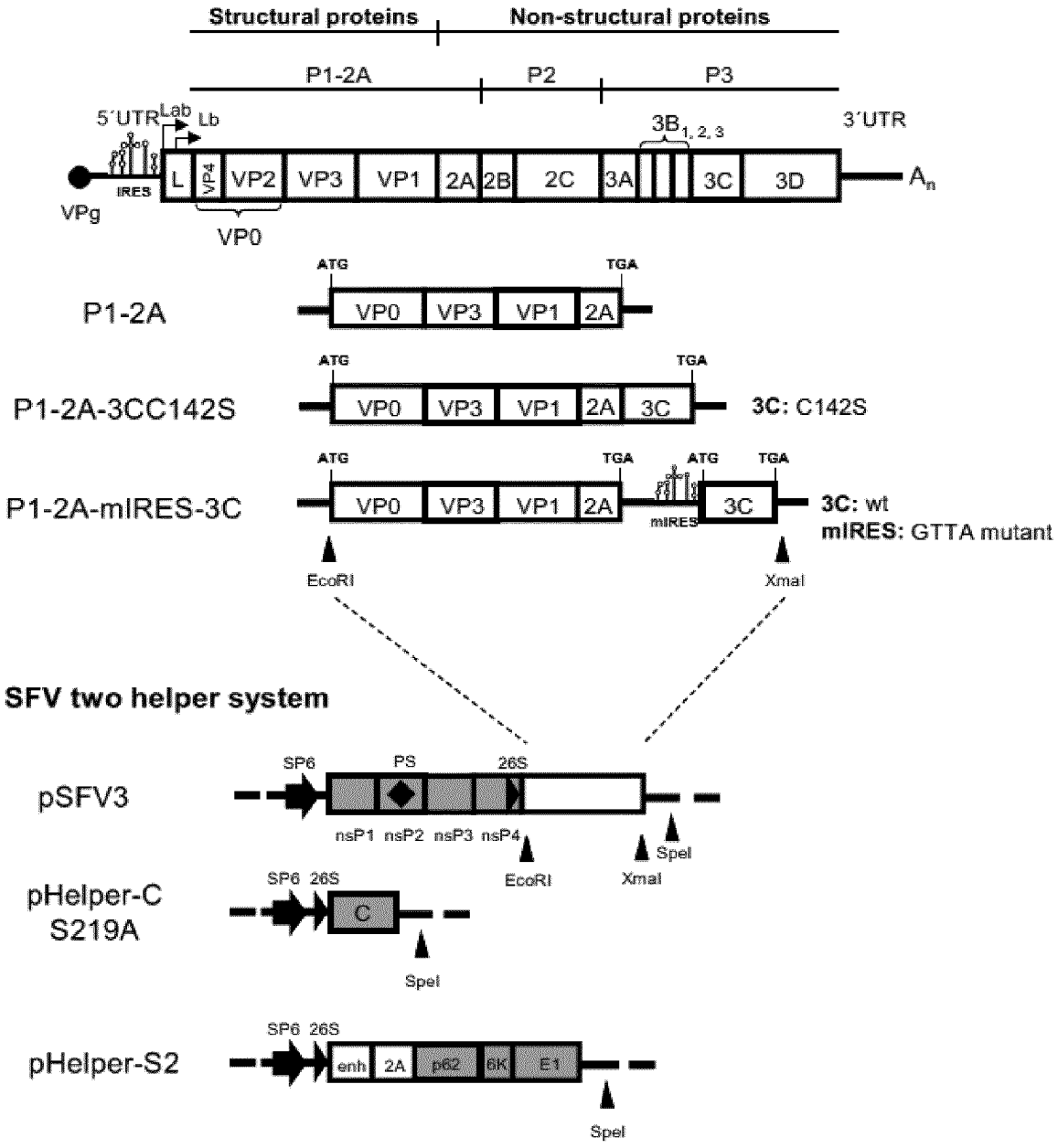
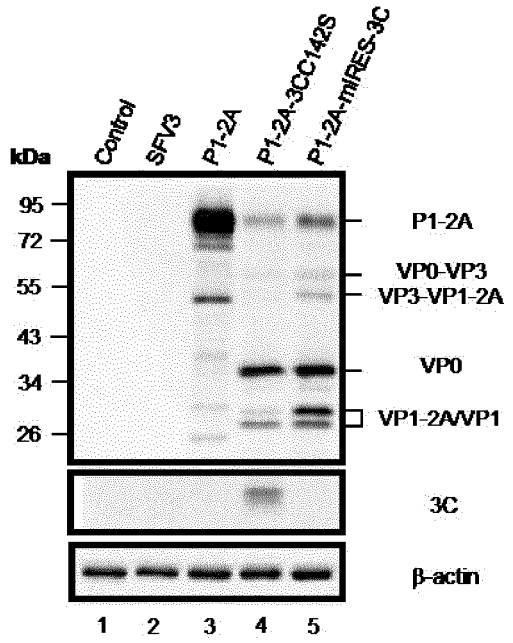
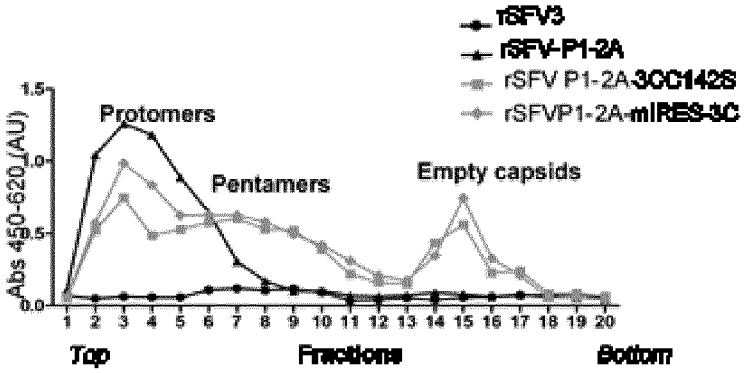

Structural proteins
Non-structural proteins
P1-2A
P2
P3
5´UTR
Lab
Lb
3B1, 2, 3
3´UTR
IRES
L
VP4
VP2
VP3
VP1
2A
2B
2C
3A
3C
3D
An
VPg
VP0
ATG
TGA
P1-2A
VP0
VP3
VP1
2A
P1-2A-3CC142S
3C
3C: C142S
P1-2A-mIRES-3C
mIRES
3C: wt
mIRES: GTTA mutant
EcoRI
XmaI
SFV two helper system
pSFV3
SP6
PS
26S
nsP1
nsP2
nsP3
nsP4
SpeI
pHelper-C S219A
C
pHelper-S2
enh
p62
6K
E1

Figure 4


kDa
Control
SFV3
P1-2A
P1-2A-3CC142S
P1-2A-mIRES-3C
95
72
55
43
34
26
P1-2A
VP0-VP3
VP3-VP1-2A
VP0
VP1-2A/VP1
3C
β-actin
1 2 3 4 5


rSFV3
rSFV-P1-2A
rSFV P1-2A-3CC142S
rSFVP1-2A-mIRES-3C
Protomers
Pentamers
Empty capsids
Abs 450-620 (AU)
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Top
Fractions
Bottom

5

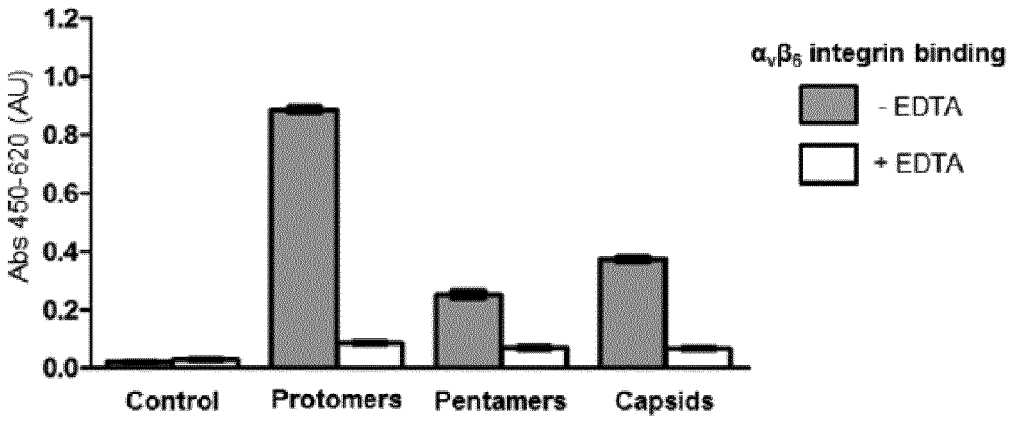

Abs 450-620 (AU)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Protomers
Pentamers
Capsids
αvβ6 integrin binding
- EDTA
+ EDTA

Figure 5

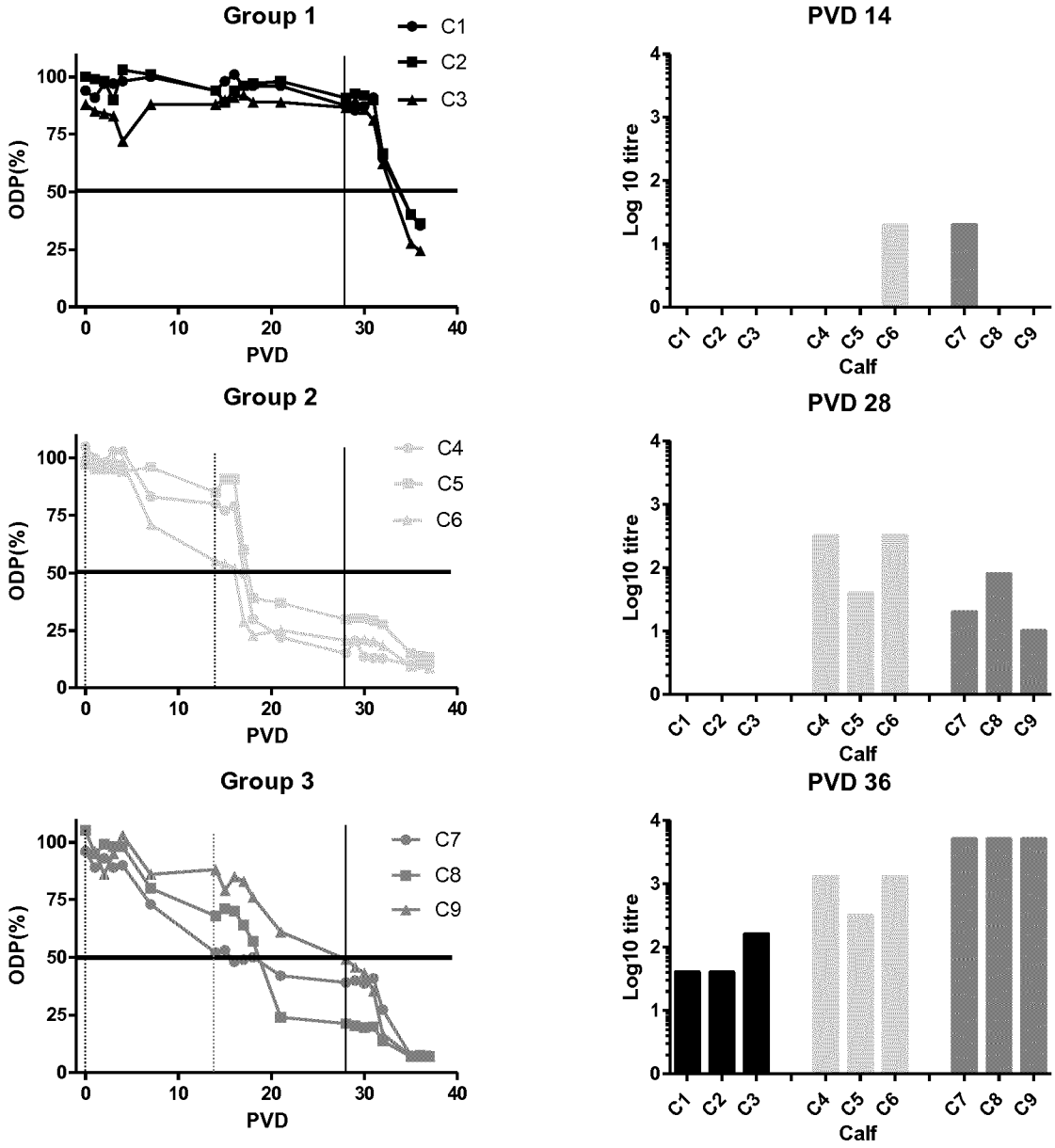

Group 1
C1
C2
C3
ODP(%)
PVD
Group 2
C4
C5
C6
ODP(%)
PVD
Group 3
C7
C8
C9
ODP(%)
PVD
PVD 14
Log 10 titre
Calf
PVD 28
Log10 titre
Calf
PVD 36
Log10 titre
Calf

Figure 6

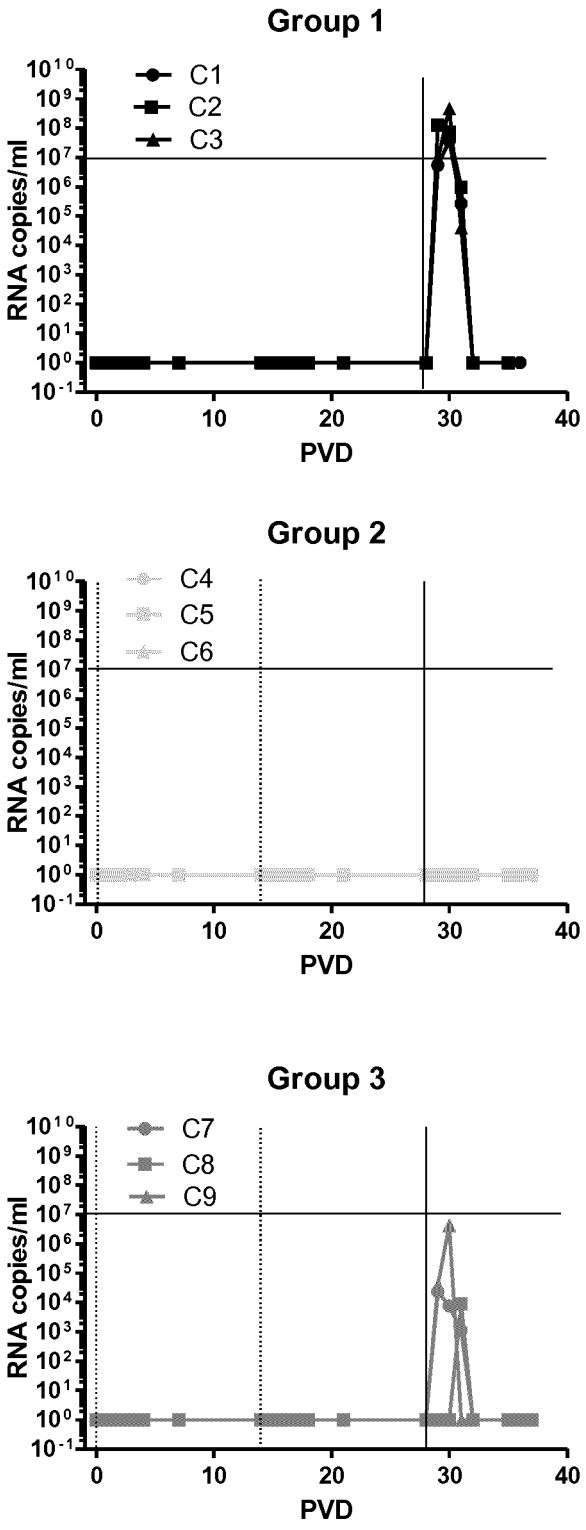
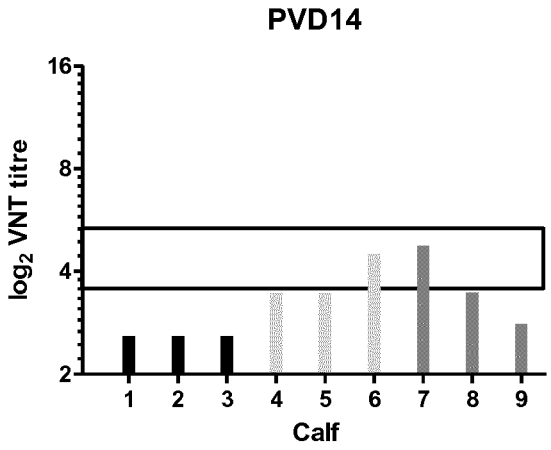
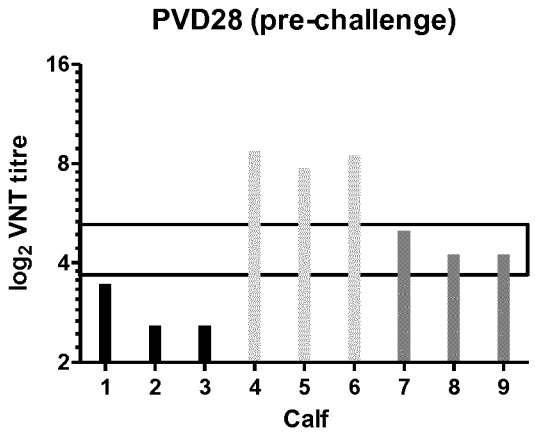
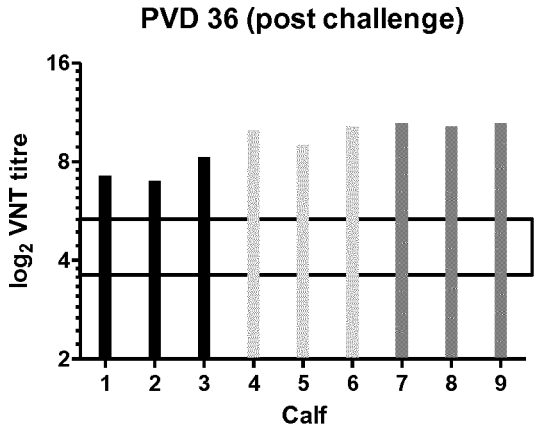

Group 1
C1
C2
C3
RNA copies/ml
PVD
Group 2
C4
C5
C6
RNA copies/ml
PVD
Group 3
C7
C8
C9
RNA copies/ml
PVD

Figure 7


PVD14
16
8
4
2
log2 VNT titre
1 2 3 4 5 6 7 8 9
Calf


PVD28 (pre-challenge)
16
8
4
2
log2 VNT titre
1 2 3 4 5 6 7 8 9
Calf

5


PVD 36 (post challenge)
16
8
4
2
log2 VNT titre
1 2 3 4 5 6 7 8 9
Calf

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/080185

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/12
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, Sequence Search, EMBASE, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	POLACEK CHARLOTTA ET AL: "Low levels of foot-and-mouth disease virus 3C protease expression are required to achieve optimal capsid protein expression and processing in mammalian cells", JOURNAL OF GENERAL VIROLOGY, vol. 94, no. Part 6, June 2013 (2013-06), pages 1249-1258, XP002754915, cited in the application abstract Discussion	1-15
A	US 2013/064839 A1 (HARRIS DELBERT LINN [US] ET AL) 14 March 2013 (2013-03-14) paragraph [0251] - paragraph [0256] paragraph [0087]	1-15

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 February 2017	15/02/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Rojo Romeo, Elena

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RAYNER J O ET AL: "ALPHAVIRUS VECTORS AND VACCINATION", REVIEWS IN MEDICAL VIROLOGY, CHICHESTER, GB, vol. 12, no. 5, 1 September 2002 (2002-09-01), pages 279-296, XP008035758, ISSN: 1052-9276, DOI: 10.1002/RMV.360 page 288, left-hand column, paragraph 3 - page 289, left-hand column, paragraph 1	1-15
A	JOHN DUSTIN LOY ET AL: "Development and evaluation of a replicon particle vaccine expressing the E2 glycoprotein of bovine viral diarrhea virus (BVDV) in cattle", VIROLOGY JOURNAL, BIOMED CENTRAL, LONDON, GB, vol. 10, no. 1, 28 January 2013 (2013-01-28), page 35, XP021139864, ISSN: 1743-422X, DOI: 10.1186/1743-422X-10-35 abstract Methods page 2, left-hand column, paragraph 2	1-15
A	XIAOLAN YU ET AL: "Enhanced immunogenicity to food-and-mouth disease virus in mice vaccination with alphaviral replicon-based DNA vaccine expressing the capsid precursor polypeptide (P1)", VIRUS GENES, KLUWER ACADEMIC PUBLISHERS, BO, vol. 33, no. 3, 1 December 2006 (2006-12-01), pages 337-344, XP019438207, ISSN: 1572-994X, DOI: 10.1007/S11262-005-0073-1 abstract	1-15
A	WO 2011/112945 A2 (MERIAL LTD [US]; BIOLEX THERAPEUTICS [US]; AUDONNET JEAN-CHRISTOPHE [F]) 15 September 2011 (2011-09-15) cited in the application page 42, line 11 - line 16 page 45, line 11 - line 32 page 39, line 21 - line 32	1-15

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CLAUDINE PORTA ET AL: "Rational Engineering of Recombinant Picornavirus Capsids to Produce Safe, Protective Vaccine Antigen", PLOS PATHOGENS, vol. 9, no. 3, 27 March 2013 (2013-03-27), page e1003255, XP055245847, DOI: 10.1371/journal.ppat.1003255 cited in the application Discussion figures 4,5	1-15

X,P	MARIA GULLBERG ET AL: "A Prime-Boost Vaccination Strategy in Cattle to Prevent Foot-and-Mouth Disease Using a "Single-Cycle" Alphavirus Vector and Empty Capsid Particles", PLOS ONE, vol. 11, no. 6, 13 June 2016 (2016-06-13), page e0157435, XP055341437, DOI: 10.1371/journal.pone.0157435 the whole document	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/080185

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2013064839 A1	14-03-2013	US 2013064839 A1	14-03-2013
		US 2013122025 A1	16-05-2013
		US 2015157703 A1	11-06-2015
WO 2011112945 A2	15-09-2011	AR 080515 A1	11-04-2012
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		CN 103687614 A	26-03-2014
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		EP 2944322 A2	18-11-2015
		EP 3093025 A1	16-11-2016
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		NZ 702145 A	31-03-2016
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		US 2011236416 A1	29-09-2011
		US 2011236420 A1	29-09-2011
		US 2017022255 A1	26-01-2017
		WO 2011112945 A2	15-09-2011
		WO 2011112955 A1	15-09-2011
		ZA 201206619 B	29-05-2013